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Veterinary Parasitology 135 (2006) 133–136
veterinary parasitology
www.elsevier.com/locate/vetpar

Biologic and molecular characterization of *Toxoplasma gondii* isolates from pigs from Portugal

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Received 22 July 2005; received in revised form 23 August 2005; accepted 24 August 2005

Abstract

Little is known of *Toxoplasma gondii* infections in animals in Portugal. In the present paper, we report the first isolation of viable *T. gondii* from pigs in Portugal. Antibodies to *T. gondii* were found in 52 (15.6%) of 333 pigs prior to slaughter using the modified agglutination test (MAT) at a serum dilution of 1:20. Attempts were made to isolate *T. gondii* from 37 seropositive pigs. Samples of brain and/or heart from each pig were digested in acid pepsin, and bioassayed into mice. Viable *T. gondii* was isolated from 15 pigs. Restriction fragment length polymorphism on products of SAG2 locus amplified by PCR and microsatellite analysis revealed that 11 isolates were Type II and four were Type III. The results indicate that phenotypically and genetically *T. gondii* are similar to isolates from pigs from the U.S.
Published by Elsevier B.V.

Keywords: *Toxoplasma gondii*; Pigs; Genotyping; Portugal; Bioassay

1. Introduction

Infection by the protozoan *Toxoplasma gondii* is prevalent worldwide in most warm-blooded hosts (Dubey and Beattie, 1988). However, only a small percentage of exposed adult humans develop clinical toxoplasmosis. It is unknown whether the severity of toxoplasmosis in immunocompetent persons is due to the parasite strain, host variability, or to other factors.

Domestic pigs (*Sus scrofa*) are considered an important source of *T. gondii* infection in humans (Dubey and Beattie, 1988) and acute toxoplasmosis has been reported in humans that have consumed uncooked infected meat from pigs (Choi et al., 1997).

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0304-4017/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.vetpar.2005.08.012

Although toxoplasmosis has been reported in humans from Portugal, little is known of *T. gondii* infections in animals. There is a single report of isolation of *T. gondii* from an aborted bovine fetus (Canada et al., 2002). The objective of the present study was to attempt isolation of *T. gondii* from pigs in Portugal.

2. Materials and methods

2.1. Naturally-infected pigs

The pigs (*Sus scorfa*) surveyed were from the Vinhais (41 50N, 7 0W) region of Portugal. They were 1–4 years old and belong to an autochthonous race of Iberian pigs (locally called porco bísaro). The animals were raised in small farms in semi-intensive management and were mainly used for sausages. They were killed in a commercial slaughterhouse, approximately 200 km from Porto, Portugal.

Blood, brain, and/or heart from 333 pigs killed between October 2004 and February 2005 were transported on ice to the laboratory in Porto. Sera were separated next day and tested for antibodies to *T. gondii* using the modified agglutination test (MAT) as described (Dubey and Desmonts, 1987) Sera were diluted twofold 1:10 to 1:80.

2.2. Bioassay of pig tissues for *T. gondii*

Tissues from 37 seropositive pigs (2 with a titer of 1:20, and 35 with titers of 1:40 or higher) were selected for isolation of *T. gondii*. For bioassay, tissues (50 g) were homogenized in five volumes (w/v) of aqueous 0.85% NaCl solution (saline), mixed with five volumes of acidic pepsin and the mixture incubated in a shaker water bath for 1 h at 37 °C (Dubey, 1998). The digest was centrifuged, neutralized with sodium bicarbonate, mixed with antibiotics, and the homogenate was inoculated subcutaneously into two Swiss Webster mice as described by Dubey (1998). Imprints of lungs and brain of the mice that died were examined for *T. gondii* tachyzoites or tissue cysts (Dubey and Beattie, 1988). Survivors were bled on day 45 post-inoculation (p.i.) and a 1:25 dilution of serum from each mouse was tested for *T. gondii* antibodies by MAT. Mice were killed 50 days p.i. and their brain squashes were examined microscopically for tissue cysts (Dubey and Beattie, 1988). Mice were considered infected with *T. gondii* when tachyzoites or tissue cysts were demonstrable in their tissues (Dubey et al., 2002).

2.3. Maintenance of *T. gondii* isolates in cell culture

The isolates were maintained by successive passages in VERO cells culture. Brains from infected mice were inoculated intraperitoneally in to two immunosuppressed mice as described by Canada et al. (2002). Tachyzoites were obtained from the peritoneal wash and mixed with a suspension of VERO cells. The cultures were maintained with Modified Eagle Medium with Earle's Modified Salts (without L-Glutamate) (Gibco, NY, USA), 10% bovine fetal serum (Gibco), 1% L-glutamate (Gibco), and 2% penicillin (5000 u/ml)/streptomycin (5000 u/ml) (Gibco). Cultures were incubated at 37 °C in a moist atmosphere with 5% CO_2. Cultures were maintained for 2–3 months in cell cultures and tachyzoites from them were sub inoculated into two Swiss Webster mice.

2.4. Genetic characterization

T. gondii DNA was extracted from infected mouse tissue or infected cell cultures using the QIAmp DNA Mini Kit (Quiagen, Courtaboeuf, France), according to the manufacturer's protocol. PCR-restriction fragment length polymorphism (RFLP) genotypes of *SAG2* locus was used to determine the genetic type (Howe et al., 1997). Additional genetic characterization was done using five microsatellite loci (*TUB2, TgM-A, W35, B17, B18*) as described by Ajzenberg et al. (2002, 2005). This multilocus analysis of microsatellite length polymorphism has a higher discriminative power than a monolocus typing by *SAG2* PCR-RFLP alone.

3. Results

Antibodies to *T. gondii* were found in 63 pigs with MAT titers of 1:10 in 11, 1:20 in 7, 1:40 in 12, and 1:80 or higher in 33 pigs. *T. gondii* was isolated from tissues of 15 seropositive pigs (Table 1). All mice inoculated

Table 1
Isolation of viable *Toxoplasma gondii* from tissues of pigs in Portugal and genotype determined by *SAG2* PCR-RFLP and a multilocus microsatellite (MS) analysis

Pig			Bioassay in mice	Isolate designation	Microsatellites (MS)					MS type	SAG2 type
Number	MAT titer	Tissue tested	No. of mice positive/no. inoculated		TUB2	W35	TgM-A	B18	B17		
44	40	Heart	2/2	TgPiPr1	2.3	2.3	2	2	2.3	II	II
116	80	Heart	2/2	TgPiPr2	2.3	2.3	3	1.3	2.3	III	III
214	80	Brain	2/2	TgPiPr3	2.3	2.3	2	2	2.3	II	II
220	80	Brain	2/2	TgPiPr4	2.3	2.3	3	1.3	2.3	III	III
227	80	Brain	2/2	TgPiPr5	2.3	2.3	2	2	2.3	II	II
231	80	Brain	2/2	TgPiPr6	2.3	2.3	2	2	2.3	II	II
232	40	Brain	2/2	TgPiPr7	2.3	2.3	2	2	2.3	II	II
238	80	Brain	2/2	TgPiPr8	2.3	5	2	2	2.3	II[a]	II
266	40	Brain	1/2	TgPiPr9	2.3	2.3	2	2	2.3	II	II
272	80	Brain	2/2	TgPiPr10	2.3	2.3	2	2	2.3	II	II
274	80	Brain	2/2	TgPiPr11	2.3	2.3	2	2	2.3	II	II
282	40	Brain	2/2	TgPiPr12	2.3	2.3	2	2	2.3	II	II
302	80	Heart	2/2	TgPiPr13	2.3	2.3	2	2	2.3	II	II
311	80	Brain/heart	2/2	TgPiPr14	2.3	2.3	3	1.3	2.3	III	III
316	20	Brain	2/2	TgPiPr15	2.3	2.3	3	1.3	2.3	III	III

[a] Atypical allele at W35 locus, see text.

with pig tissues survived except one mouse that died of toxoplasmosis; this mouse had been inoculated with tissues from pig no. 238. All *T. gondii* isolates were successfully grown in cell culture. Subpassage of tachyzoites from culture from two isolates (from pigs 116 and 311) killed Swiss Webster mice within 9–12 days p.i. and tachyzoites were found in their lungs; these isolates had been maintained in cell culture for 2–3 months before inoculation in to mice. *T. gondii* tissue cysts were found in the brains of mice that survived for 2 months after inoculation with cell cultures.

SAG2 and microsatellite typing indicated that 11 isolates were Type II, and 4 were Type III (Table 1). One isolate (PV238) had an atypical allele (5) for the *W35* locus, the length of the amplified product containing the microsatellite sequence, measured by GeneScan analysis, being 244 bp, instead of 242 bp usually observed for classical Type II allele at this locus.

4. Discussion

In the present study, *T. gondii* was isolated from 15 (47.5%) of seropositive pigs, which is much lower than 63–98% isolation rate from pigs from USA (Dubey et al., 1995a, 2002). These findings are probably due to type of bioassay used (cats versus mice) and the number of mice used (10 versus 2 mice), and the amount of tissue sampled (50 g versus 100 g). Therefore, these results are not a reflection of the sensitivity of the serologic test used. The MAT has been found to be the most specific and sensitive test to detect *T. gondii* infection in pigs (Dubey et al., 1995b; Dubey, 1997; Gamble et al., 2005). The antibody titer that should be considered indicative of latent infection in pigs is not certain because viable *T. gondii* has been isolated from 3 to 4% of seronegative pigs (Dubey et al., 1995a, 2002). Therefore, we presented data on all pigs, even though we consider a MAT titer of 1:25 as indicative of persistent *T. gondii* infection in pigs, based on bioassays of naturally-infected pigs (Dubey et al., 1995a, 2002).

T. gondii isolates have been classified into three genetic Types (I, II, III) based on restriction fragment length polymorphism (RFLP) of six loci (Howe and Sibley, 1995; Boothroyd and Grigg, 2002). Type I strains are considered virulent for mice whereas Types II and III were considered avirulent. A monolocus *SAG2* PCR-RFLP typing is generally considered as sufficient to classify the isolates in to one of the three main genotypes. However, this concept is changing because the strains from Brazil are virulent, irrespective of the *SAG2* genotype (Dubey et al., 2002). In the present study, the genotypes obtained by *SAG2* PCR-RFLP were confirmed by a multilocus analysis with five microsatellites. The atypical allele observed for one microsatellite locus (*W35)* for PV238 isolate does not modify the belonging of this isolate to the Type II lineage. Phenotypically and genetically, the porcine isolates from pigs in Porto were like those from the United States, and different from those from isolates from pigs from Brazil. In the present study, of the 15 *T. gondii* isolates from pigs from Portugal, 11 were Type II, and 4 were Type III and 13 of 15 isolates were avirulent for mice. Of the 43 of 170 *T. gondii* isolates from sows in Iowa selected for *SAG2* genotyping; 36 isolates were Type II, and 7 were Type III (Dubey et al., 1995; Mondragon et al., 1998). Of the 25 isolates from finisher pigs from a farm in USA (Dubey et al., 2002) selected for *SAG2* typing, 20 were Type III and 5 were Type II (Lehmann et al., 2003). None of isolates from pigs in USA was mouse virulent. Of the seven isolates of *T. gondii* from pigs in Brazil five were Type III, and two were Type I; all were virulent for mice (dos Santos et al., 2005). In the present study the isolates from pigs in Portugal were generally avirulent for mice.

Acknowledgements

The authors thank to Dr. Alcina Rodrigues for his assistance in obtaining samples from the pigs and IBERLAB. IMUNOREAGE and BIOGNOSIS for facilities.

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